The Use of Short Term Tests to Evaluate Progress toward Reducing the Toxicity of Cigarette Smoke

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Introduction

A Risk Reduction Strategy. Reynolds Tobacco has for decades actively pursued the development of cigarettes with the potential to reduce the risks associated with smoking. We have pioneered a number of techniques to reduce overall "tar" and nicotine yields, and we have developed technologies to reduce specific compounds and classes of compounds in cigarette smoke (R. J. Reynolds, 1988).

In conjunction with these efforts, the company has developed or applied a number of sophisticated chemical and biological assays to evaluate the effects of its cigarette developments. These assays and their results have frequently been presented at national and international scientific meetings and published in peer-reviewed journals (list of publications available upon request).

In light of the fact that some 50 million American adults and between 6.5 and 7 million Canadian adults choose to smoke and that tens of millions will, without doubt, continue to do so in the years ahead, Reynolds Tobacco believes that:

1) Adults should have the legal right to make informed choices among a full range of cigarettes, balancing the pleasures and risks associated with smoking.
2) A regulatory framework should be designed that allows free-market forces to encourage the development of, and the dissemination of appropriate information about cigarettes and cigarette alternatives that have the potential to reduce the risks associated with smoking.
3) Smokers should be given accurate information concerning performance differences between and among individual cigarette brands, including differences that might have the potential to reduce risks.

R. J. Reynolds' efforts to develop cigarettes with the potential to reduce the risks associated with smoking have been guided by a simple, intuitive approach: "Less ought to be better." That is, reductions in tobacco smoke constituents should result in reductions in the risks associated with the use of cigarettes and other tobacco products. That philosophy has also been the foundation for recommendations from the scientific community and a variety of governments during the past 40-plus years.

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Based on learning obtained from a long history of efforts, R.J. Reynolds believes that the following approaches offer the greatest opportunity for developing cigarettes that have the potential to reduce the risks associated with smoking and that will be accepted by smokers:

1. Continuing to pursue general and specific smoke constituent reductions in tobacco-burning cigarettes.
2. Continuing to develop and refine alternative cigarette designs (such as heating tobacco) that offer substantial general reductions not achievable through design techniques traditionally use for cigarettes.
3. Finding ways to alter "tar"/nicotine ratios, to achieve additional general "tar" reductions.

At the same time, our efforts will continue to ensure that no product developments or modifications add to the inherent biological activity of smoke from cigarettes currently on the market.

Perhaps the most difficult aspect of developing cigarettes with the potential to reduce the risks associated with smoking is determining how to measure progress toward that goal. Despite many decades of debate, the scientific community still lacks consensus concerning what cigarette modifications might lead to reductions in risk and how those reductions should be measured and characterized.

Those who would require long-term epidemiological studies before acknowledging that a cigarette might have the potential to reduce risk would create a situation where 20 to 30 years of evaluation would be required before product innovations could be communicated or introduced (Wright, 1998). We believe that preventing the introduction of such products until evidence of this type is available represents a poor public health approach and would stifle, rather than promote, the development of new cigarettes – a result that is inconsistent with the approaches taken for pharmaceuticals, foods or other products.

Instead, we believe that efforts should be made to reach a consensus on logical and practical standards that must be met to allow the marketing (with appropriate claims) of potentially reduced risk products. Such standards might include, for example, a tiered toxicological evaluation that may, on some occasions, incorporate short-term human clinical studies, with appropriate clinical surrogate markers. In addition, a way to monitor the use and certain effects of the marketed product over time might also by included in these standards.

Tiered Testing Approach. Cigarette design has evolved over a century with the incorporation of many new technologies in the last several decades (Jones and Norman, 1998). Modifications to cigarette design have historically involved the incorporation of new ingredients, tobacco processes, papers, and filters which has the potential to modify the quantity and quality of the smoke yielded from the cigarette. RJRT continues to develop and evaluate novel technologies.

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RJRT has developed a tiered testing strategy to evaluate the potential for new ingredients, tobacco processes, and technological developments to increase or reduce the biological activity of our products. This tiered testing strategy was created to provide a conceptual framework based on level of concern to facilitate the design of toxicological evaluation programs. Within the context of this framework, decisions regarding the design of a toxicological evaluation program are determined by considering two factors – the level of human exposure and the potential for toxicity. The level of human exposure reflects not only the level (frequency and magnitude) of expected exposure but also the intended use level in the cigarette. The potential for toxicity is determined based upon available information (structure activity relationships, *in vitro* data, and *in vivo* data). All data, including positive and negative findings, are considered to derive a weight-of-the-evidence conclusion. Combined, these factors, in conjunction with scientific judgment, provide a means to assess the appropriate level of concern. In brief, the level of concern is a relative measure of the extent to which a product modification may present a potential risk or a potential to present a reduced risk.

Based upon the level of concern, an appropriate toxicological evaluation program is designed. For example, a modification determined to represent a low level of concern (such as a subtle change in the formulation of a cigarette paper) may require no significant toxicological evaluation. By contrast, a modification determined to represent a high level of concern such as the introduction of novel technology exemplified by PREMIER and ECLIPSE may require more extensive toxicological evaluation. The concern level attributed to these cigarettes is a direct reflection of the uniqueness of the technology employed as well as the significant potential to reduce cigarette smoke toxicity (R. J. Reynolds, 1988). Such an evaluation would include an examination of a number of smoke constituents (which have been identified by the Surgeon General and others to have the potential to contribute to the risks associated with smoking) as well as both *in vitro* and *in vivo* toxicology.

Benchmarking. At RJRT comparative studies typically constitute the foundation of the toxicological evaluation programs designed to address the potential of new technology to reduce the biological activity associated with burning tobacco. In general the comparative approach is based on a benchmark established using data generated on either a reference cigarette (such as the University of Kentucky reference cigarettes) or a survey of the marketplace. Market survey data is generated by obtaining and evaluating a representative sample (typically based on market share) of the cigarette brands in commerce (Steele *et al.*, 1994; Swauger *et al.*, 1997).

The utility of the Kentucky reference cigarettes as representative benchmarks of the cigarette market has been previously described (Steele *et al.*, 1994; Swauger *et al.*, 1997). Therefore, It is appropriate to use these reference cigarettes as a benchmark in comparative studies designed to characterize the potential of new technologies to reduce the biological activity associated with burning tobacco.

Market survey data provide a useful tool to extend the conclusions drawn in the direct head to head comparative studies to the broader context of the marketplace. Prior studies of smoke composition (constituent yield) and associated toxicity (Ames activity) have demonstrated that current cigarettes are generally similar. Constituent yields and Ames activity are in general predicted by 'tar' yield. These data indicate that differences in product (exemplified by the

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and II alveolar epithelial cells may result in the release of cytokines responsible for the recruitment of macrophages (Driscoll *et al.*, 1990; Sibille and Reynolds, 1990; Wolff *et al.*, 1990; Nathan *et al.*, 1980). Damage to Type I and II cells may also cause genetic changes or an abnormal proliferative response of these cells within the alveoli (Driscoll *et al.*, 1994; Bond *et al.*, 1990; Witschi, 1976). Several of the pulmonary cell types (e.g., Clara cells, alveolar macrophages, etc.) have significant metabolizing capability which can activate chemicals to a more cytotoxic form (Chichester *et al.*, 1991; Forkert *et al.*, 1989; Rietjens *et al.*, 1988; Domin *et al.*, 1986; Devereux, 1984; Jones *et al.*, 1982; Devereux *et al.*, 1981). Chemicals which injure these various pulmonary cell types to induce a pulmonary inflammation may also be associated with chronic disease processes such as emphysema and cancer (Phan and Thrall, 1995; Toyokuni *et al.*, 1995; Yeadon, 1995; Kamp *et al.*, 1992; Kovacs, 1991; Shaw *et al.*, 1991; Haslett and Henson, 1988; Fedullo and Ettensohn, 1985; Crystal *et al.*, 1984; Devereux, 1984; Swenberg *et al.*, 1983; Jones *et al.*, 1982; Brody, 1981; Hunninghake *et al.*, 1979).

Tests to Measure Genetic Toxicology

Properly validated "short *term*" *in vitro* genetic toxicology tests provide an invaluable tool for new product development and evaluation. These tests are primarily used as screens to measure the potential for DNA damage by examining bacteria or mammalian tissue culture cells in the laboratory. The foundation for this approach is derived from observations and studies indicating that the induction of many cancers can be traced to mutational exposures resulting from chemical exposures. (Sawyer, 1994). *In vitro* genetic toxicology assays are useful in setting priorities for further testing, and they may play a key role in product development decisions. These short-term tests provide an alternative to animal testing and are used extensively worldwide. They can be conducted quickly and efficiently at relatively low cost.

Various members of the scientific community draw different conclusions concerning the applicability of the results from these types of tests to human risk assessment. Some scientists believe that these tests can predict whether exposure to test substances will result in adverse effects in humans, while others do not agree, arguing that results from tests of non-human cells, organs, or species may not be directly applicable to human beings. However, there is general agreement within the scientific community that these assays are valuable in a weight of the evidence approach to toxicological evaluations.

New guidelines for a standard battery of genotoxicity tests have been recently recommended by the "International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use" (ICH). The ICH guidelines state, "Genotoxicity tests can be defined as *in vitro* and *in vivo* tests designed to detect compounds which induce genetic damage directly or indirectly by various mechanisms...Registration of pharmaceuticals requires a comprehensive assessment of their genotoxic potential. It is clear that no single test is capable of detecting all relevant genotoxic agents."

Two of the three assays recommended by ICH in the standard test battery are *in vitro* assays: 1) a test for gene mutation in bacteria; 2) an *in vitro* test with cytogenetic evaluation of chromosomal damage with mammalian cells or an *in vitro* mouse lymphoma tk assay. We have modified this test battery for our specific needs at RJRT. The Ames *Salmonella* Assay is the

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Examples include ethanol toxicity and liver cirrhosis (Colton and Zakhari, 1997; Kurase *et al.*, 1996; Bondy, 1992), chloroform toxicity and liver necrosis (Plaa, 1997; Cheeseman *et al.*, 1985; Trivedi and Mowat, 1983; Klaasen and Plaa, 1969), and paraquat toxicity and decreased lung function (Krall *et al.*, 1988; Bus and Gibson, 1984).

There are several examples that have been reported in the literature that illustrate the importance of cytotoxicity and cellular injury to a pathological process. Several of these examples are described below:

Dermal Initiation/Promotion Assay in the Mouse (Mouse Skin Painting Assay)

The mouse skin painting assay examines the potential of a chemical or chemical mixture to induce skin tumors in the mouse. This assay has been used frequently as an animal test to assess the carcinogenic potential of chemicals (Boutwell, 1989; Slaga, 1986; Boutwell *et al.*, 1981). Some of the chemicals that are positive in this assay are cytotoxic and have been identified as tumor "promoters." The cellular injury induced by these chemicals result in the proliferation of cell populations that may have a mutated phenotype and ultimately produce a malignant tumor. The classic tumor promoting agent, phorbol 12-myristate 13-acetate is cytotoxic and causes the generation of reactive oxygen species, skin inflammation, and a skin hyperplasia (Kensler *et al.*, 1989; Cerutti, 1985), all of which are mechanisms previously described as possible outcomes from cytotoxicity. Other cytotoxic chemicals including benzoyl peroxide, mezerein, and canthardin cause similar effects in skin and are skin tumor promoters in the mouse skin painting assay (Digiovanni *et al.*, 1987; O'Connell *et al.*, 1987; Slaga *et al.*, 1983; Slaga *et al.*, 1981).

Examples of the Role Cytotoxicity May Play in Atherosclerosis

Several reports in the literature illustrate the involvement of cellular injury to the potential development of atherosclerotic plaques. Endothelial cells within the pulmonary system are directly susceptible to reactive oxygen species present in the lung (Cross, 1987) which could lead to compromised pulmonary vasculature. Also, oxidized low density lipoprotein formed by macrophages has been reported to be cytotoxic to endothelial cells (Mehra *et al.*, 1995; Dzau, 1994). The initial damage to arterial endothelial cells by a cytotoxic mechanism may be the initiation of the sclerotic process involving formation of arterial fatty streaks, deposition of foam cells, and the ultimate manifestation of an atherosclerotic plaque (Dzau, 1994; O'Brien and Chait, 1994). In addition, thrombogenic events can incur cellular damage to endothelial cells to result in a similar arterial lesion (Dzau, 1994; Nachman and Silverstein, 1993; Rubanyi, 1993).

Examples of the Role Pulmonary Inflammation May Play in Lung Disease

Several lines of evidence suggest that pulmonary inflammation derived from cytotoxic events leads to pathological sequelae. Pulmonary macrophages are an important pulmonary cell type with a key role in mediating the inflammatory response and maintaining the integrity of the lung parenchyma (Fels and Cohn, 1986). Macrophages activated or killed by chemicals (Oosting *et al.*, 1990; Graham and Gardner, 1985; Sone *et al.*, 1983; Gardner and Graham, 1976) or particles (Dixit *et al.*, 1990; Bowden, 1987; Allison, 1974) can secrete or release proteolytic enzymes and reactive oxygen species which can injure surrounding pulmonary cells and result in an inflammatory response (Nathan *et al.*, 1980; Harris *et al.*, 1970). Similarly, damage to Type I

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Short-term tests can broadly be viewed as measuring two major endpoints: cytotoxicity and genotoxicity. Cytotoxicity is regarded as an important step in several chronic disease processes associated with smoking, including carcinogenesis and emphysema. These tests are also very helpful for determining the appropriate exposure concentration for *in vitro* genotoxicity assays. Importantly the use of *in vitro* cytotoxicity assays minimizes the use of animals for toxicity assessment. The major endpoints evaluated in *in vitro* cytotoxicity assays in our laboratory and others include the effect of a test agent on cell viability and the effect of a test agent on cellular growth rates. Decreases in viability and/or growth rates are widely interpreted as evidence of cytotoxicity. Genetic toxicology, on the other hand, looks specifically at the effects of the chemical on the DNA molecule. The purpose of genetic toxicology studies is to characterize and define the potential of test agents to adversely affect the structure or function of DNA molecules. Genotoxicity has been mechanistically linked with carcinogenesis, both in experimental animals and in humans. Also, DNA damage in germ cells may produce heritable defects in offspring and DNA damage has been associated with the development of arteriosclerosis.

Cytotoxicity Assays

Tests to Assess Cytotoxicity. Cytotoxicity examines the toxic effects of chemical or physical agents on cellular structural or functional components. Biological endpoints for cytotoxicity may examine general mechanisms common to all cells (e.g. DNA synthesis, mitochondrial function) or mechanisms specific to a particular cell type (e.g. hormone secretion, metabolizing enzymes, neurotransmitter release) A list of general cytotoxicity biological endpoints is given in Table 1 (adapted from Balls and Clothier, 1992).

Table 1

Endpoint	Endpoint Measurement
Cell morphology	Cell size and shape Cell-cell contacts Nuclear size, shape, and inclusions Nuclear number, size, shape, and inclusions Cytoplasmic vacuolization
Cell viability	Vital dye uptake (e.g., neutral red) Trypan blue exclusion Cell number Replating efficiency
Cell adhesion	Attachment to culture surface Cell-cell adhesion
Cell proliferation	Increase in cell number Increase in total DNA Increase in total RNA Increase in total protein Colony formation
Membrane damage	Loss of enzymes (e.g., LDH) Loss of ions or cofactors (e.g., Ca, K, NSDPH)

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diversity of the marketplace) generally do not alter either composition of cigarette smoke or the activity of cigarette smoke condensate on a specific activity basis.

As indicated, the available data demonstrate that the composition of smoke and the resulting toxicity of current cigarettes are generally similar. Therefore it is unnecessary to completely characterize the marketplace on a brand by brand basis in order to soundly demonstrate the risk-reduction potential of new technology. The potential of new technology to reduce the biological activity associated with burning tobacco may be effectively evaluated in terms of the existing cigarette brands through the use of market surveys. Taken as a whole, this approach can be effectively employed on a weight-of-the-evidence basis to measure progress toward reducing the biological activity associated with burning tobacco.

A comparative approach for measuring progress with cigarettes which have the potential to reduce risk against cigarettes currently on the market has been referred to as ***the Rule of First Approximation*** (Rose, 1998). This approach would not require a series of clinical studies (Phase I through Phase IV) similar to the testing required by the U.S. FDA to make therapeutic drug claims. This more pragmatic approach would rely on the weight of the evidence from chemistry and toxicology compared to a suitable benchmark of marketed cigarettes to justify short-term clinical tests, which rely on surrogate markers. If progress can be demonstrated using this comparative approach, than appropriate claims about the potential for risk reduction would be permitted. This approach would permit a more rapid introduction of modified cigarettes into the market and realization of the benefit of reduced risk products.

Following is a review of the in vitro tests that have been used to evaluate cigarette smoke and cigarette smoke condensate. Our company and other consumer products companies have used these short-term tests to construct tiered toxicological evaluations appropriate to each individual product change. It must be emphasized that not all product changes would undergo testing using these assays depending on the level of concern.

Short-term *In Vitro* Assays

Short-term *in vitro* assays are ideally suited to the tiered testing strategy for measuring progress towards the reduction of cigarette smoke toxicity. They are widely accepted by regulatory agencies around the world, are fairly inexpensive and rapid to conduct, and there is an excellent mechanistic basis for believing that the endpoints measured in these tests are causally related to the risk of developing chronic disease. Short-term tests are amenable to studying a multitude of cigarette products and/or cigarette components. Smoke from new cigarette prototypes, cigarettes from the U.S. market, or Kentucky reference cigarettes may easily be studied. Whole cigarette smoke as well as condensate or other subfractions of the whole smoke are easily evaluated for toxicity in short-term tests. Additionally, one may examine the toxicity of sidestream smoke, mainstream smoke or environmental tobacco smoke in these assays. These assays are also extremely useful for assessing the toxicity of isolated components in cigarette smoke, such as additives and flavors, as well as studying how the complex cigarette smoke mixture may affect the toxicity of isolated components or chemicals found in tobacco smoke.

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vapor and particulate fractions (Bombick *et al.*, 1997; Bombick *et al.*, 1991). The cytotoxicity of mainstream and sidestream smoke from various reference cigarettes were compared and exposure of cell cultures using this in vitro exposure system. In addition, this exposure system was used to expose cell cultures to a high level of environmental tobacco smoke (approximately 1.5 mg TPM/m³) with the result of no cytotoxicity observed in the exposed cell cultures (Bombick *et al.*, 1991).

Cytoxicity Assessment of Innovative Cigarettes. The cytotoxic potential of mainstream smoke was assessed from two recently developed cigarettes exhibiting reduced smoke chemistries. A cigarette with a novel carbon filter exhibiting a significant reduction in vapor phase irritants (e.g., formaldehyde, acetaldehyde, acrolein, isoprene, etc.) also demonstrated a significant reduction in cytotoxicity of the whole smoke when compared to the smoke from a similar cigarette without the carbon filter (Bombick *et al.*, 1997). Another cigarette that primarily heats tobacco exhibited significant reductions of chemicals when compared to tobacco burning cigarettes (Borgerding *et al.*, 1998). The smoke condensate from the cigarette which heats tobacco exhibited no cytotoxicity as shown in Figure 1 (Bombick, D. W. *et al.*, 1998; Bombick, B. R., 1998; Bombick *et al.*, 1996; Bombick *et al.*, 1995). The whole smoke from the tobacco-heating cigarette had similar cytotoxicity to an ultra-low "tar" reference cigarette and significantly reduced cytotoxicity compared to a light "tar" reference cigarette (Figure 2).

Relationship of Cytotoxicity Assessments to In Vivo Toxicity Assessments. The significance of cytotoxicity to *in vivo* situations can be separated into four broad areas indicated below:

- Irritation and inflammation
- Cell proliferation and hyperplasia
- Oxidative stress and damage
- Decreased organ function

All these processes are dependent upon a cytotoxic injury which if chronically maintained can lead to a pathological condition. These processes may also initiate compensatory mechanisms that will lead to recovery or in some situations increase susceptibility of the animal or human to disease. The correlation of cytotoxicity with irritation and inflammation can be observed in several organ systems including skin (Roguet, 1997; Roguet *et al.*, 1994; Gueniche and Ponec, 1993; Cohen *et al.*, 1991), eye (Spielmann, 1997; Balls *et al.*, 1995; Osborne *et al.*, 1995), cardiovascular system (Ohno *et al.*, 1994; Rossi *et al.*, 1994; Roberts, 1995; Schaefer *et al.*, 1995), and pulmonary system (Li *et al.*, 1986; Saladino *et al.*, 1985; Li *et al.*, 1983). Cell injury can induce cell proliferation and tissue hyperplasia as responses to inflammation (Stadnyk, 1994; Albelda *et al.*, 1993; Argyris, 1989; Luger *et al.*, 1985), wound healing (Border and Noble, 1994; Gailit and Clark, 1994; Stadnyk, 1994), and liver regeneration (Mehendale *et al.*, 1994; Mehendale, 1991). Cytotoxicity and cellular injury that result in oxidative damage and the release of reactive oxygen species to other tissues can induce inflammatory (Varani and Ward, 1997; Varani and Ward, 1994; Halliwell, 1992) and hyperplastic responses (Butterworth and Goldsworthy, 1991; Amstad *et al.*, 1990; Schulte-Herman *et al.*, 1990). The necessity for tissues to reduce the oxidative stress can decrease normal physiological processes including metabolism (Somani *et al.*, 1997; Aruma, 1994). The release of reactive oxygen species may also lead to direct genetic damage and increased potential for mutations (Guyton and Kensler, 1993; Breimer, 1990; Floyd, 1990). In severe situations, cytotoxicity can destroy an organ system.

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	Leakage from preloaded cells (e.g., vital dye)
Uptake or incorporation of radioactive precursors	Thymidine and DNA synthesis Uridine and RNA synthesis Amino acids and protein synthesis
Metabolic effects	Inhibition of metabolic cooperation Cofactor depletion (e.g., ATP) Mitochondrial function

Role of Cytotoxicity Assays in Industry and Government Toxicity Assessments. The use of cytotoxicity assays, as part of the toxicological assessment of a chemical or chemical mixture is currently a very active area of consideration. Several large research groups have investigated the role of cytotoxicity assays in toxicology and have made recommendations to industrial and government bodies. In the United States the Center for Alternatives to Animal Testing (CAAT) at Johns Hopkins University have recommended several cytotoxicity tests for the safety testing of consumer products, such as cosmetics, detergents, and other personal hygiene products (Balls *et al.*, 1990). A large multi-national effort entitled MEIC (multi-center evaluation of in vitro cytotoxicity) have examined a large number of cytotoxicity endpoints in a variety of cell types using a standardized list of 50 diverse chemicals with varying mechanisms of action. The MEIC studies have indicated a very good correlation with cytotoxicity and acute lethal potency and irritant potential (Ekwall *et al.*, 1994; Clothier *et al.*, 1989; Ekwall *et al.*, 1989). Another large research program, FRAME (fund for the replacement of animals for medical experimentation) has identified several cytotoxicity assays including the neutral red test for the toxicological assessment of chemicals and potential pharmaceuticals (Faotrel *et al.*, 1991; Knox *et al.*, 1986; Rowan and Stratman, 1980). Cytotoxicity assays are now being considered to replace the Draize eye test and the OECD guideline no. 405 for acute eye irritation/corrosion has been modified for this accommodation. With respect to cosmetics, Directive 76/768/EEC, article 4 has prohibited the marketing of products that have been tested on animals as of January 1998 therefore, requiring in vitro tests including cytotoxicity assays. Considerable effort has been made to validate the use of cytotoxicity assays for toxicity assessments. A summary of scientific criteria for the validation of in vitro toxicity testing has been compiled by CAAT, FRAME, and OECD (Frazier, 1992; FRAME, 1991, Balls *et al.*, 1990; Organization for Economic Cooperation and Development, 1990).

Cytotoxicity Assessments of Cigarette Smoke and Cigarette Smoke Components. A number of our studies have been conducted to optimize assays and conditions for the cytotoxicity assessment of cigarette smoke and cigarette smoke components. The neutral red uptake assay has been chosen from a number of cytotoxicity assays as being the most sensitive assay for assessing the cytotoxic potential of cigarette smoke condens es (Putnam *et al.*, 1998; Bombick and Doolittle, 1992). The neutral red assay has been optimized and used for cytotoxicity assessments comparing cigarette smoke condensates from cigarettes differing in "tar" amounts or different tobacco types (Bombick *et al.*, 1998; Putnam *et al.*, 1997). The cytotoxicity of specific chemicals reported in cigarette smoke have been determined in the neutral red assay (Bombick and Doolittle, 1995) and recently, several phenolic and dihydroxybenzenes have been shown to have minimal contribution to the cytotoxicity of the entire cigarette smoke condensate mixture (Bombick *et al.*, in press). The neutral red assay also has been used to validate an in vitro exposure system that permits exposure of cell cultures to the entire cigarette smoke comprised of

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and II alveolar epithelial cells may result in the release of cytokines responsible for the recruitment of macrophages (Driscoll *et al.*, 1990; Sibille and Reynolds, 1990; Wolff *et al.*, 1990; Nathan *et al.*, 1980). Damage to Type I and II cells may also cause genetic changes or an abnormal proliferative response of these cells within the alveoli (Driscoll *et al.*, 1994; Bond *et al.*, 1990; Witschi, 1976). Several of the pulmonary cell types (e.g., Clara cells, alveolar macrophages, etc.) have significant metabolizing capability which can activate chemicals to a more cytotoxic form (Chichester *et al.*, 1991; Forkert *et al.*, 1989; Rietjens *et al.*, 1988; Domin *et al.*, 1986; Devereux, 1984; Jones *et al.*, 1982; Devereux *et al.*, 1981). Chemicals which injure these various pulmonary cell types to induce a pulmonary inflammation may also be associated with chronic disease processes such as emphysema and cancer (Phan and Thrall, 1995; Toyokuni *et al.*, 1995; Yeadon, 1995; Kamp *et al.*, 1992; Kovacs, 1991; Shaw *et al.*, 1991; Haslett and Henson, 1988; Fedullo and Ettensohn, 1985; Crystal *et al.*, 1984; Devereux, 1984; Swenberg *et al.*, 1983; Jones *et al.*, 1982; Brody, 1981; Hunninghake *et al.*, 1979).

Tests to Measure Genetic Toxicology

Properly validated "short term" *in vitro* genetic toxicology tests provide an invaluable tool for new product development and evaluation. These tests are primarily used as screens to measure the potential for DNA damage by examining bacteria or mammalian tissue culture cells in the laboratory. The foundation for this approach is derived from observations and studies indicating that the induction of many cancers can be traced to mutational exposures resulting from chemical exposures. (Sawyer, 1994). *In vitro* genetic toxicology assays are useful in setting priorities for further testing, and they may play a key role in product development decisions. These short-term tests provide an alternative to animal testing and are used extensively worldwide. They can be conducted quickly and efficiently at relatively low cost.

Various members of the scientific community draw different conclusions concerning the applicability of the results from these types of tests to human risk assessment. Some scientists believe that these tests can predict whether exposure to test substances will result in adverse effects in humans, while others do not agree, arguing that results from tests of non-human cells, organs, or species may not be directly applicable to human beings. However, there is general agreement within the scientific community that these assays are valuable in a weight of the evidence approach to toxicological evaluations.

New guidelines for a standard battery of genotoxicity tests have been recently recommended by the "International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use" (ICH). The ICH guidelines state, "Genotoxicity tests can be defined as *in vitro* and *in vivo* tests designed to detect compounds which induce genetic damage directly or indirectly by various mechanisms...Registration of pharmaceuticals requires a comprehensive assessment of their genotoxic potential. It is clear that no single test is capable of detecting all relevant genotoxic agents."

Two of the three assays recommended by ICH in the standard test battery are *in vitro* assays: 1) a test for gene mutation in bacteria; 2) an *in vitro* test with cytogenetic evaluation of chromosomal-damage with mammalian cells or an *in vitro* mouse lymphoma tk assay. We have modified this test battery for our specific needs at RJRT. The Ames *Salmonella* Assay is the

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Examples include ethanol toxicity and liver cirrhosis (Colton and Zakhari, 1997; Kurase *et al.*, 1996; Bondy, 1992), chloroform toxicity and liver necrosis (Plaa, 1997; Cheeseman *et al.*, 1985; Trivedi and Mowat, 1983; Klaasen and Plaa, 1969), and paraquat toxicity and decreased lung function (Krall *et al.*, 1988; Bus and Gibson, 1984).

There are several examples that have been reported in the literature that illustrate the importance of cytotoxicity and cellular injury to a pathological process. Several of these examples are described below:

Dermal Initiation/Promotion Assay in the Mouse (Mouse Skin Painting Assay)

The mouse skin painting assay examines the potential of a chemical or chemical mixture to induce skin tumors in the mouse. This assay has been used frequently as an animal test to assess the carcinogenic potential of chemicals (Boutwell, 1989; Slaga, 1986; Boutwell *et al.*, 1981). Some of the chemicals that are positive in this assay are cytotoxic and have been identified as tumor "promoters." The cellular injury induced by these chemicals result in the proliferation of cell populations that may have a mutated phenotype and ultimately produce a malignant tumor. The classic tumor promoting agent, phorbol 12-myristate 13-acetate is cytotoxic and causes the generation of reactive oxygen species, skin inflammation, and a skin hyperplasia (Kensler *et al.*, 1989; Cerutti, 1985), all of which are mechanisms previously described as possible outcomes from cytotoxicity. Other cytotoxic chemicals including benzoyl peroxide, mezerein, and canthardin cause similar effects in skin and are skin tumor promoters in the mouse skin painting assay (Digiovanni *et al.*, 1987; O'Connell *et al.*, 1987; Slaga *et al.*, 1983; Slaga *et al.*, 1981).

Examples of the Role Cytotoxicity May Play in Atherosclerosis

Several reports in the literature illustrate the involvement of cellular injury to the potential development of atherosclerotic plaques. Endothelial cells within the pulmonary system are directly susceptible to reactive oxygen species present in the lung (Cross, 1987) which could lead to compromised pulmonary vasculature. Also, oxidized low density lipoprotein formed by macrophages has been reported to be cytotoxic to endothelial cells (Mehra *et al.*, 1995; Dzau, 1994). The initial damage to arterial endothelial cells by a cytotoxic mechanism may be the initiation of the sclerotic process involving formation of arterial fatty streaks, deposition of foam cells, and the ultimate manifestation of an atherosclerotic plaque (Dzau, 1994; O'Brien and Chait, 1994). In addition, thrombogenic events can incur cellular damage to endothelial cells to result in a similar arterial lesion (Dzau, 1994; Nachman and Silverstein, 1993; Rubanyi, 1993).

Examples of the Role Pulmonary Inflammation May Play in Lung Disease

Several lines of evidence suggest that pulmonary inflammation derived from cytotoxic events leads to pathological sequelae. Pulmonary macrophages are an important pulmonary cell type with a key role in mediating the inflammatory response and maintaining the integrity of the lung parenchyma (Fels and Cohn, 1986). Macrophages activated or killed by chemicals (Oosting *et al.*, 1990; Graham and Gardner, 1985; Sone *et al.*, 1983; Gardner and Graham, 1976) or particles (Dixit *et al.*, 1990; Bowden, 1987; Allison, 1974) can secrete or release proteolytic enzymes and reactive oxygen species which can injure surrounding pulmonary cells and result in an inflammatory response (Nathan *et al.*, 1980; Harris *et al.*, 1970). Similarly, damage to Type I

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Because bacteria lack many of the metabolic capabilities of mammals, an exogenous metabolic activation system was developed to enable conversion of promutagens to mutagens in *in vitro* assays. This metabolic activation system consists of a tissue homogenate and cofactor mix; the tissue homogenate used most often is a 9000 x *g* supernatant (S9) of rat liver (Claxton *et al.*, 1987; Maron and Ames, 1983). The Ames test is usually conducted under two conditions - with and without the addition of S9 metabolic activation.

The first reports of Ames assays of cigarette smoke were published in 1974 (Kier et al) and 1975 (Hutton and Hackney). Cigarette smoke condensate, the particulate matter of mainstream smoke, is mutagenic in most of the basic Ames tester strains in the presence of S9 metabolic activation, and particularly so in TA98, a strain that measures frameshift mutations. More recently developed *Salmonella* strains enhanced with *O*-acetyltransferase activity (YG1024, YG1029) have proven to be especially sensitive to mutagens in cigarette smoke (De Flora *et al.*, 1995). The mutagenicity of CSC is influenced by many factors: tobacco type (for example, burley vs flue-cured), stalk position, age, sugar and nitrogen content of tobacco leaves (deMarini, 1983). The mutagenicity is affected by the pressure drop, filters, and other physical characteristics (Sato *et al.*, 1977). The Ames assay is therefore a sensitive and very useful assay in product development and toxicity evaluation of cigarette smoke, and data from RJRT laboratories have been published (Steele *et al.*, 1995).

The Ames assay can also be used as a convenient and non-invasive way of evaluating recent human exposure to potentially genotoxic chemicals. The vast majority of studies to evaluate human exposure to mutagens have been conducted on urine specimens (Rynard, 1990). These so called urinary mutagenicity studies have been used to assess environmental, dietary and occupational exposures; for example, methods of food preparation can significantly affect urinary mutagenicity (Doolittle *et al.*, 1989). RJRT has conducted urinary mutagenicity studies to compare cigarettes which burn or primarily heat tobacco (Doolittle *et al.*, 1989; Smith *et al.*, 1996). These studies concluded that urine of smokers of cigarettes that primarily heat tobacco (Premier and Eclipse) is significantly less mutagenic that urine of smokers of tobacco-burning cigarettes.

Chromosome Aberration Assay. The chromosome aberration assay measures structural changes and rearrangements in chromosomes resulting from damage to genetic material. These can be analyzed in cultured cells. Mammalian cells represent a higher degree of organization than bacteria with markedly different DNA repair, xenobiotic metabolism, and other functions (Sawyer, 1994), and the inclusion of a mammalian cell assay lends another dimension to a short-term test battery.

Aberrations are a consequence of failure or mistakes in repair processes after damage has been done. The chromosomes are unable to rejoin, or they rejoin in abnormal configurations. Aberrations may be lethal or may be transmitted through repeated cell divisions. The assay is scored by examining chromosomes in cells under a microscope and recording the number of chromosome aberrations in each cell. Since many aberrations are lethal, cytogenetic evaluations have to be conducted in a period of time shortly after exposure when the cells are in first post-

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cornerstone of short term testing at RJRT and is used extensively for product evaluation. We use the Sister Chromatid Exchange Assay (SCE Assay) as well as the Chromosome Aberration Assay as part of our testing strategy. (The SCE assay is used for comparative purposes to a greater extent than the Chromosome Aberration assay).

ICH guidelines recommend a third short-term *in vivo* test for chromosomal damage using rodent hematopoietic cells. In vivo bone marrow studies of rodents exposed to cigarette smoke have reported consistent in vivo genotoxic effects. Mice are the only rodent species reported to have cytogenetic alterations following exposure to cigarette smoke, and then only after exposure of high doses of high-tar cigarettes (Reed *et al.*, 1994). We conducted extensive tests at RJRT evaluating sister chromatid exchange and micronuclei, which confirmed reports that rodents are refractory to these types of in vivo genotoxic changes. A 90-day subchronic inhalation study in rats exposed to mainstream cigarette smoke did not show cytogenetic effects in bone marrow as measured by SCE, micronucleus, and chromosome aberration assays (Lee *et al.*, 1990a). These rats were exposed subchronically to high doses of mainstream smoke, as indicated by high blood carboxyhemoglobin concentrations (COHb), high plasma nicotine concentrations, and histopathological changes in the respiratory tract (Reed *et al.*, 1994). A 14-day inhalation study in mice showed no significant increases in SCE or chromosome aberrations. A marginal response in micronuclei was observed at the highest dose only; statistical analyses indicated that this increase may be interpreted as either significant or not, depending on the statistical test applied (Reed *et al.*, 1994; Coggins *et al.*, 1990). Therefore this third test—an *in vivo genotoxicity assay*-- has not proven to be useful in the evaluation of cigarette smoke.

Ames Salmonella Mutagenicity Assay. Bacterial mutagenicity assays have been used in research laboratories and by regulatory agencies for more than two decades (Josephy, 1997). These assays are highly sensitive for genotoxins and are carried out with relative technical ease, rapidity, and economy (Sawyer, 1994).

Gene mutations may contribute to human disease through several pathways. An increase in the mutation rate of human germ cells may cause an increased incidence in heritable genetic disease while mutations in somatic cells may contribute to various disorders, including cancer (Hoffmann, 1996). Mutations may play a role in carcinogenesis, and may activate oncogenes or inactivate or delete tumor suppressor genes (Hoffmann, 1996). Thus information from microbial mutation assays has proven to be a valuable research tool.

The Ames assay is generally the first step in a standard short-term test battery. It is the most widely used genetic toxicology test. The Ames assay measures the ability of a test article to produce a mutation in specific strains of Salmonella bacteria that have been modified to be extremely sensitive to mutagens. The Ames assay measures point mutations and each tester strain has specific sensitivities to different types of point mutations.

The Ames assay is a reverse mutation assay. In the presence of a mutagen, the bacteria revert to their normal ability to synthesize histidine (i.e., wild type) and therefore grow on an agar plate which contains only minimal nutrients (Maron, 1983). An increase in revertants is a measure of the mutagenicity of a test substance. Response in the Ames assay is measured by scoring bacterial colonies on agar plates that cover a range of dose concentrations.

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Tucker and Preston name several advantages of SCE assays: Sister chromatid exchanges can be scored relatively easily and quickly; the assay yields quantifiable data from every cell; and the genotoxic potential of a compound can be determined more rapidly than with some other cytogenetic methods. Data from the SCE assay is amenable to statistical analysis, and large SCE databases have been generated. SCE's are generally a more sensitive indicator of genotoxic effects than are structural aberrations. There are clearly both significant advantages and disadvantages associated with this assay.

The SCE assay is, however, an excellent biomarker for cigarette smoke, and is complementary to the Ames assay. Tobacco smoke condensate is generally positive in the Ames assay primarily with metabolic activation. Cigarette smoke condensate is clearly positive in the SCE assay with and without metabolic activation. The SCE assay is also sensitive not only to the particulate phase of cigarette smoke but also to vapor phase components as well. Cigarette smoke induces SCE's in a dose-dependent manner, both in the particulate and the vapor phase. The SCE assay is sensitive to both direct and indirect mutagens in cigarette smoke at low concentrations. The SCE assay (as well as chromosome aberration assay) is therefore sensitive to direct-acting components of CSC that are not detected in the Ames assay. (DeMarini, 1983). Margarete Curvall and associates had several publications in the mid-80s reporting on their work with fractions of CSC in the SCE and Ames assays (Curvall, *et al.*, 1985; Jansson *et al.*, 1986; Curvall and Enzell, 1984).

In vitro genotoxicity assays have been widely used to study the biological activity of tobacco smoke and tobacco smoke condensate (DeMarini, 1983; IARC, 1986). RJRT has published several papers reporting results of *in vitro* genetic toxicology assays on tobacco smoke condensates and new products (Doolittle *et al.*, 1990a; Doolittle *et al.*, 1990b; Lee *et al.*, 1990; Steele *et al.*, 1995; Bombick B.R. *et al.*, 1998; Bombick, D.W. *et al.* 1998). RJRT has also conducted urinary mutagenicity studies comparing cigarettes which burn or primarily heat tobacco (Smith *et al.*, 1996). *In vitro* genotoxicity assays are ideally suited to the testing strategy for measuring progress towards the reduction of cigarette smoke toxicity.

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treatment mitosis. Evaluation of aberrations requires specialized training, and is a laborious process "requiring considerable patience and expertise" (Sawyer, 1994).

An excellent review paper was published in 1996 describing "the four cytogenetic endpoints most frequently used in hazard identification assays as the first step in the risk assessment process" (Tucker & Preston, 1996). One of those assays is the chromosome aberration assay. Tucker and Preston state that there is a clear relationship between chromosome rearrangements and cancer (Solomon 1991, Rabbitts, 1994, Mitelman, 1994) with virtually all tumors containing structural chromosome rearrangements. The authors agree that the associations between chromosome alterations and the development of tumors and birth defects enhance their utility as a relevant endpoint in short term mutagenicity assays.

The relationship between chromosomal aberrations and tumorgenesis is one justification for including cytogenetic endpoints in toxicological evaluations and new product development, environmental monitoring, assessment, cleanup and for workplace hazard evaluations (Tucker & Preston, 1996).

Cigarette smoke condensate has the potential to induce chromosome aberrations in cultured cells, both in the presence and absence of metabolic activation. Lafi and Perry (1991)reported that total particulate matter (TPM) generated from cigarettes with different ventilation characteristics yielded chromosome aberrations, which varied according to the ventilation of the cigarette. RJRT has published several papers reporting positive results in chromosome aberration assays of cigarette smoke condensate. Some of these studies compared chromosome aberrations in cigarette smoke condensate from cigarettes that burn versus cigarettes that primarily heat tobacco. Cigarettes that primarily heat tobacco show a great reduction in the potential to induce chromosomal aberrations (Doolittle, *et al.*, 1990a; Doolittle *et al.*, 1990b; Lee *et al.*, 1990b; Bombick *et al.*, 1998).

Sister Chromatid Exchange Assay. The sister chromatid exchange (SCE) assay evaluates the potential of a test article to increase the exchange of genetic material between sister chromatids in a chromosome. Sister chromatids exchange genetic material even under normal circumstances. An increase in exchanges is thought to be an indicator of damage to the DNA. The assay is scored by examining the chromosomes in cells and scoring the number of exchanges between sister chromatids in a cell.

Tucker and Preston's review paper addresses the utility of SCE assays in short term toxicity assessments. SCE assays have been commonly employed to evaluate cytogenetic responses to chemical exposure, and excellent dose-response relationships for hundreds of chemicals have been established in a wide variety of in vitro and in vivo short-term experiments (Tucker 1993b). However, the mechanisms of SCE formation have not been established, and the biological significance of sister chromatid exchanges (SCE's) is not understood. Because of this and the fact that there is no direct association between SCE induction and an adverse cellular or health outcome, their analysis has limited value in risk assessment except as a biological dosimeter or biomarker of exposure to genotoxic chemicals (Tucker & Preston, 1996). A positive SCE response does not necessarily mean that a compound is mutagenic (for example, sodium chloride and potassium chloride have been shown to induce SCE (Tucker & Preston, 1996)).

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